Microarray analysis of gene expression in eastern oyster (Crassostrea virginica) reveals a novel combination of antimicrobial and...

Article *in* Fish & Shellfish Immunology · December 2010

DOI: 10.1016/j.fsi.2010.07.035 · Source: PubMed

CITATIONS
50

READS
117

12 authors, including:

David Bushek
Rutgers, The State University of New Jersey
107 PUBLICATIONS **1,606** CITATIONS
SEE PROFILE

Yongping Wang
Rutgers, The State University of New Jersey
56 PUBLICATIONS **641** CITATIONS
SEE PROFILE

Ximing Guo
Rutgers, The State University of New Jersey
210 PUBLICATIONS **5,326** CITATIONS
SEE PROFILE

Zhanjiang Liu
Auburn University
413 PUBLICATIONS **8,398** CITATIONS
SEE PROFILE

Fish & Shellfish Immunology 29 (2010) 921–929

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Microarray analysis of gene expression in eastern oyster (*Crassostrea virginica*) reveals a novel combination of antimicrobial and oxidative stress host responses after dermo (*Perkinsus marinus*) challenge

Shaolin Wang [a], Eric Peatman [a], Hong Liu [a], David Bushek [b], Susan E. Ford [b], Huseyin Kucuktas [a], Jonas Quilang [a,c], Ping Li [a], Richard Wallace [a], Yongping Wang [b], Ximing Guo [b], Zhanjiang Liu [a,*]

[a] *The Fish Molecular Genetics and Biotechnology Laboratory, Department of Fisheries and Allied Aquacultures and Program of Cell and Molecular Biosciences, Aquatic Genomics Unit, 203 Swingle Hall, Auburn University, Auburn, AL 36849, USA*
[b] *Haskin Shellfish Research Laboratory, Institute of Marine and Coastal Sciences, Rutgers University, 6959 Miller Ave., Port Norris, NJ 08349, USA*
[c] *Institute of Biology, College of Science, University of the Philippines, 1101 Diliman, Quezon City, Philippines*

ARTICLE INFO

Article history:
Received 18 June 2010
Received in revised form
15 July 2010
Accepted 26 July 2010
Available online 12 August 2010

Keywords:
Oyster
Microarray
Dermo
Oxidative stress
Antimicrobial

ABSTRACT

Dermo disease, caused by *Perkinsus marinus*, is one of the most severe diseases of eastern oysters, *Crassostrea virginica*. It causes serious mortalities in both wild and aquacultured oysters. Using existing expressed sequence tag (EST) resources, we developed a 12K *in situ* oligonucleotide microarray and used it for the analysis of gene expression profiles of oysters during the interactions between *P. marinus* and its oyster host. Significant gene expression regulation was found at day 30 post-challenge in the eastern oyster. Putative identities of the differentially expressed genes revealed a set of genes involved in several processes including putative antimicrobial defenses, pathogen recognition and uptake, anti-oxidation and apoptosis. Consistent with results obtained from previous, smaller-scale experiments, expression profiles revealed a large set of genes likely involved in an active mitigating response to oxidative stress and apoptosis induced by *P. marinus*. Additionally, a unique galectin from *C. virginica*, CvGal, which serves as a preferential receptor for *P. marinus* trophozoites, was found to be significantly down-regulated in gill tissue of oysters with both light and heavy infection, suggesting an attempt to control parasite uptake and proliferation in the later stages of infection. Potential histone-derived antimicrobial responses to *P. marinus* were also revealed in the gene expression profiles.

1. Introduction

The eastern oyster, *Crassostrea virginica*, is an economically important species. It is widely fished and cultured in the Gulf of Mexico and along the eastern coast of the United States [1]. Oysters also serve as a fundamental component of the aquatic ecosystem by creating habitat and removing phytoplankton and silt from the water column [2]. The eastern oyster has been used as a marine bivalve model to study the effects of environmental stressors [3] and as a bioindicator of estuarine pollution [4,5]. However, eastern oyster populations have been threatened by disease as well as overfishing and habitat degradation [6,7]. Epizootics of dermo disease have become one of the major threats to oysters because they result in massive mortalities in both wild and aquaculture populations [8].

Dermo disease (also known as Perkinsosis) is caused by *Perkinsus marinus*, a protozoan parasite belonging to the Alveolates [9,10]. As infections develop in eastern oysters, *P. marinus* causes reduction in shell growth, decreased hemolymph protein concentrations and lysozyme activities, severe emaciation, inhibition of gonadal development and reproductive output, and ultimate death [11]. In spite of the devastating impact caused by the parasite [12], the mechanisms of pathogenicity and the physiological and defense responses of the host are still poorly understood [13].

One of the better studied aspects of host responses to *P. marinus* infection is the oxidative stress response. During infection, large quantities of cytotoxic oxidants are released, such as reactive oxygen species (ROS), which are responsible for eliminating some parasite species [14]. However, the ROS released in response to the parasite is converted to hydrogen peroxide (H_2O_2) by SOD, which can increase levels of other highly toxic species, such as hydroxyl radical (OH), or hydroxylchlorous acid [15]. These reactive intermediates cause oxidative stress in oysters. In mammalian systems, severe oxidative stress can cause cell death and necrosis while moderate oxidative

* Corresponding author. Tel.: +1 334 844 8727; fax: +1 334 844 9208.
E-mail address: liuzhan@auburn.edu (Z. Liu).

1050-4648/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fsi.2010.07.035

stress can trigger apoptosis [16]. Apoptosis induced by *P. marinus* infection has been reported in the oyster [17,18]. In order to reduce the damage caused by oxidative stress and apoptosis, the host can generate anti-oxidant and anti-apoptotic agents, such as metallothioneins, which display oxyradical scavenging capacity and can reduce oxidative stress [19,20].

Additional avenues of research on oyster immune responses have focused on the roles of lectins in pathogen recognition and handling. Several lectins have been identified from *Crassostrea gigas* [21], but did not show an induction in synthesis following bacterial infection. More relevant to this study, Tasumi and Vasta [22] have reported that a unique *C. virginica* galectin, CvGal, serves as a preferential hemocyte receptor for *P. marinus*, providing passive access to the host tissues and circulatory system via phagocytosis.

Recent development and application of microarray technology has allowed rapid progress in understanding gene expression after various treatments in several finfish and shellfish species [23–25]. For oysters, a number of genome resources have been developed recently, including a large number of polymorphic markers [26–29], construction of a framework genetic linkage map [30], construction of large-insert BAC libraries [31], initial analysis of expressed sequence tags (EST) [32–37], and construction of a first-generation cDNA microarray [23]. The application of these resources is growing. For example, a cDNA microarray was used recently to study differences in tolerance to heat shock among selectively bred lines of Pacific oysters [38]. Here we report the development of a 12K oligonucleotide microarray for the eastern oyster and application of this array to study the host response at the transcriptional level of the eastern oyster after challenge by *P. marinus*. Our results serve to reinforce and extend previous observations of oxidative stress/apoptosis-driven host responses while providing novel observations of the broader innate immune response.

2. Materials and methods

2.1. Experimental oysters, disease challenge and sampling

The eastern oyster strain, NEH, was employed in the challenge (Rutgers University). The cohort used in our challenge experiment was produced, and reared to 16 months of age, on Martha's Vineyard, Massachusetts, where dermo is less prevalent than at the Rutgers rearing site in New Jersey. Shell height of the oysters ranged from 32.2 to 104.7 mm with a mean of 69.4 mm (±14.2 sd). Because *P. marinus* is present in Massachusetts, a Time 0 sample of 23 oysters was examined prior to the challenge. All oysters were acclimated to experimental conditions over a period of one week by slowly adjusting temperature and salinity to 25 °C and 25 ppt. Oysters were fed a mixture of *Isochrysis galbani* and *Thalassiosira weissflogii* during acclimation and throughout the experimental challenge, which was conducted in the Haskin Shellfish Research Laboratory.

The *P. marinus* parasites used for the challenge were obtained from naturally infected native Delaware Bay oysters as described by Ford et al. [39]. On 24th October 2006, 60 oysters were each challenged with an average of 1.11 (±0.15) × 10^5 parasites g^{-1} wet tissue weight injected into the shell cavity through a notch at the ventral margin of the valves. Wet tissue weight was estimated from a linear regression of wet tissue weight against whole animal weight in a subsample representing the size distribution of the challenged oysters ($N = 6$, $r^2 = 0.88$). Control oysters ($n = 10$) received shell cavity injections of 0.22-μm filtered seawater (FSW). Injection volumes ranged from 20 to 90 μl, depending on the size of the oyster, for both challenged and control groups.

The challenged and control oysters were held in separate recirculating water tables at 25 ppt and 25 °C with 25% water changes performed thrice weekly. No mortality occurred during the experiment and on day 30 post-inoculation, 50 oysters in each treated group were sampled. At both sampling dates, each oyster was shucked aseptically to collect mantle and rectal tissues for incubation in Ray's Fluid Thioglycollate Medium (RFTM) [40], and gill tissue for the microarray. Tissues incubated in RFTM were assayed for *P. marinus* infection intensity and ranked as described by Choi et al. on a scale from 0 to 5 in increments of 0.33 [41]. Gill tissues (50 mg) from each treated and control oyster were immediately soaked in RNAlater stabilizing buffer (Qiagen, Valencia, CA) and stored at −80 °C. Gill tissue from all 27 oysters sampled on day 30, including nine control oysters, nine oysters with light infections and nine with heavy infections were shipped overnight on dry-ice to Auburn University for the microarray study.

2.2. RNA extraction and cDNA conversion

The 9 samples from each group (control, day 30 heavy infections and day 30 light infections) were combined by grouping into 3 pools of 3 individuals each for a total of 3 pools for each treatment and control). RNA was extracted from the pooled tissue samples. The tissue samples were first homogenized in TRIzol (Invitrogen, Carlsbad, CA) by passing the lysate several times through a 20-gauge needle according to the protocol provided by the company. TRIzol extraction was applied twice to purify the total RNA. Approximately 20 μg of total RNA was obtained from each pooled tissue sample. The RNA quality and concentration were checked by spectrophotometric analysis and gel electrophoresis. All extracted samples had an A260/280 ratio of greater than 1.8, and were diluted to a final concentration of 1 μg/μl for cDNA synthesis. Total RNA was converted to double stranded cDNA using a SuperScript II cDNA synthesis kit (Invitrogen, Carlsbad, CA) and an oligo-dT primer containing the T7 RNA polymerase promoter. The cDNA quality and concentration were checked using a spectrophotometer. The cDNA quality and concentration was also checked with a Bioanalyzer (Agilent Technologies, Palo Alto, CA) by NimbleGen (Madison, WI), and only when the cDNA passed quality control (A260/280 ratio of greater than 1.8), was it used for further microarray hybridization.

2.3. Microarray fabrication, hybridization and image acquisition

A high-density *in situ* oligonucleotide microarray (oligoarray) was constructed using a unique set of ESTs assembled from 14,560 eastern oyster EST sequences and 4618 Pacific oyster EST sequences. We also included 179 *P. marinus* nucleotide sequences on the array. All of the 19,357 sequences were downloaded from GenBank and assembled into clusters using CAP3 [42] to obtain unique sequences. A total of 12,222 unique sequences was obtained, all of which were used to construct the oyster *in situ* oligonucleotide microarray. All unique transcripts were compared by BLASTX against the non-redundant (nr) protein database at NCBI, with a cutoff E-value of 1e-5 for annotation, in order to determine their putative identities.

NimbleGen designed the oyster oligoarrays using an *in situ* maskless array synthesis technology [24]. Sixty base pair (60-mer) oligonucleotides were synthesized on the surface of the microarray slides in triplicate with each unique oyster sequence represented by 10–12 60-mers. The cDNA labeling followed by array hybridization, washing, and scanning steps were all carried out by NimbleGen. A total of 9 oyster oligo microarrays, one for each control and challenged pool, was used in this experiment.

2.4. Microarray data analysis

The raw image data were first converted to gene expression values and normalized to remove non-biological variation [43,44]. All statistical analyses were conducted using dchip software [45].

Table 1
Primers used for quantitative real-time RT-PCR.

Target gene	Accession	Forward	Reverse
Elongation factor 1α (control)	AB122066	ACATTGCTCTGTGGAAGTTCG	ATGGTGGTTCAGTGCTGTCC
Cyclin B3	CD646472	CTCCTGTAGGCGTCGTCACAG	CCATGAGACACTGTACCTGGCTG
TNF receptor member 19	CD649717	ACATCAGTTGTAAAGCAGGCCTCG	TACGCACAGATGCTTTGTGGTGG
Carbonic anhydrase precursor	CD649975	TGAGAGTTCGGCGCTGTAGTC	AGGAGACTGTCGGACCAAGC
Metallothionein ivc	CV087794	TTGCAGCAACAGTTCTCGTC	CACTTGTGCCTGCGCAACAAC
Ubiquitin-conjugating enzyme	CV088129	GGAATCTTCATCAGATCCAAGTGG	GACAACAGGGGGATCTTGTGG
C-type lectin 10	CV088207	TGTGCAGTTCTGTCTCGGTC	TGATGTCTTGCGTCAACCCA
C-type lectin 1	CV088356	TCACGCTCCTCTCGGTGCTG	GCTGGTTGTCAACGGTTACCAGG
Conserved hypothetical protein	CV132138	GATAATGAGGGTCAAGGTCG	GCTGTTGTCGTCCTTCATCC
Metallothionein iie	CV133159	ACAGCCGATTGTATCACAGACAC	TGGCATTTTCAGAGGTGCCGCT
Peroxiredoxin 6	EH644464	GAGACAGTTGGTGCATCCTG	CTTTGACCAGCCCTGGTGAC
Dynein intermediate chain 1	EH647818	TCCCGCATATCAAAGGCCAC	AACCTCCACAGAGGCAGCATGG
Unknown	EH648615	ATGATGAAGACACTGGCCTG	ATTCTCTGTCAACCGTTCTG

The following criteria were used to determine the differential regulation of oyster genes: (i) greater than 100 absolute expression values (the signal intensity indicative of the expression level), (ii) 2-fold or greater change in expression between control and treatment groups ($P < 0.05$) and (iii) a global false discovery rate (FDR) of 10%. The significantly regulated transcripts were searched against the nr protein database to obtain their putative identity with cutoff E-value (1e-5).

2.5. Quantitative real-time PCR analysis

Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) was conducted to assess the microarray data. Primers were designed with the FastPCR program [46] for 12 differentially expressed genes as initially identified by microarray analysis (Table 1). One-step qRT-PCR was carried out using the RNA Master SYBR Green I reagent kit on a LightCycler instrument (Roche Applied Science, Indianapolis, IN) following the manufacturer's instructions with modifications. Briefly, all qRT-PCR reactions were performed in a 10-μl total reaction volume (9 μl master mix and 1 μl [100 ng] RNA template). The master mix contained 4.3 μl H_2O, 0.6 μl $Mn[OAc]_2$, 0.3 μl of each primer (0.1 μg/μl), and 3.5 μl of the SYBR Green mix. The same cycling parameters were used for all tested genes: (i) reverse transcription, 20 min at 61 °C; (ii) denaturing, 30 s at 95 °C; (iii) amplification repeated 50 times, 5 s at 95 °C, 5 s at 56 °C, 20 s at 72 °C; (iv) melting curve analysis, 5 s at 95 °C, 15 s at 65 °C, then up to 95 °C at a rate of 0.1 °C/s; (v) cooling, 30 s at 40 °C [24]. The triplicate (biological) fluorescence intensities of the control and treatment products for each gene, as measured by crossing-point (Ct) values, were compared and converted to fold differences by the relative quantification method using the Relative Expression Software Tool 384 v. 1 (REST) assuming 100% efficiencies [47]. Expression differences between control and challenged groups were assessed for statistical significance using a randomization test in the REST software. The mRNA expression levels of all samples were normalized to the levels of the elongation factor 1 alpha (EF-1α) [38,48–50]. EF-1α was also selected as a reference gene because it was stably expressed in *C. gigas* exposed to a variety of conditions in other studies [38,50].

Table 2
P. marinus infection statistics for control and challenged oysters assayed by the RFTM method of Choi et al. (1989). Mean intensity, which ranges from 0 to 5, is the mean infection score of all oysters in the sample, including those scored as "0". One oyster in the control group showed signs of infection at the day 30 sampling point.

Sample	N	Prevalence	Mean Intensity (sd)	Intensity Range
Control	9	11%	0.07 (0.22)	0.00–0.67
30 day Light infection	9	98%	1.34 (0.23)	0.00–2.00
30 day Heavy infection	9	96%	3.96 (0.11)	3.67–4.67

3. Results

3.1. P. marinus infection

After 30 days, the infected oysters were divided into light infection (score = 0 to 2.0) and heavy infection (score = 3.67 to 4.67) groups based on infection intensity (Table 2). Both light infection and heavy infection groups had similar prevalence (percentage of oysters infected): 98% and 96% respectively. Both groups were successfully infected and each group developed a wide range of infection intensities by day 30 group (light infection 1.34, ±0.23 SD; heavy infection 3.96, ±0.11 SD) (Table 2). We assume that oysters that developed heavy infections after 30 days were more susceptible than those that developed only light infections.

3.2. Gene expression profiles based on the 12K oligo microarray and validation

Based on the criteria selected for microarray analysis (see above), significant differences in gene expression were observed at day 30 post-challenge between the challenged and control oysters. At day 30, 807 and 545 genes were found to be differentially expressed when individuals with light or heavy infections, respectively, were compared to controls (Table 3). However, significant expression differences were not observed between heavy and light infection groups, potentially due to sample variation within the heavy and light infection replicate individuals (Table 3; Fig. 1).

A total of 12 genes were selected to validate the microarray results using quantitative reverse-transcription PCR (qRT-PCR). The qRT-PCR results confirmed, in general, the patterns of up-regulated and down-regulated expression of the 12 genes as indicated by the microarray analysis (Table 4), although the exact level of regulation varied somewhat as previously reported with catfish infected with *Edwardsiella ictaluri* [24,25].

3.3. Genes differentially expressed at 30 days after P. marinus challenge

The major objective of sampling at this time point in the study was to identify the differential expression of genes related to

Table 3
Number of up- and down-regulated genes detected by microarray analysis at 30 days after challenge of oysters with *P. marinus*.

Control	Treatment	Up-regulated	Down-regulated	Total
control	30d heavy infection	185	360	545
control	30d light infection	299	508	807

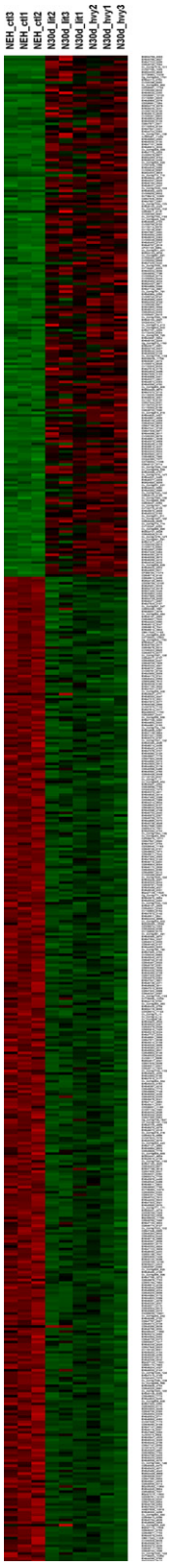

Fig. 1. Hierarchical cluster of differentially expressed genes between control and 30 day treatment groups, exhibiting reproducibility among samples. Patterns of down-regulated genes (lower panel, from red to green) and up-regulated genes (upper panel, from green to red) can be seen from the hierarchical clusters. The samples (from left to right) listed are control group 3, 1, and 2; 30 day light infection group 2, 3, and 1; heavy infection group 2, 1, and 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

disease responses. Although oysters were dosed with the same weight-standardized number of parasites, they developed a range of infection intensities during the 30-day period. A large number of genes were differentially expressed 30 days after infection although different genes were differentially expressed as compared to the control in heavily and lightly infected samples. A total of 807 genes and 545 genes were found to be regulated in lightly infected and heavily infected oysters, respectively, of which 319 genes (Supplementary Table 1) were commonly regulated in both the heavily infected and the lightly infected groups.

Of the 319 genes regulated in both groups, 76 were up-regulated, and 243 were down-regulated genes. The 319 commonly regulated genes account for 58.5% of the 545 regulated genes within heavily infected group and 39.6% of the 807 regulated genes within the light infected group. The gene function was annotated through the AmiGO database [51]. Of the commonly regulated 319 genes in both light and heavy infection oyster groups, many were unknown in their identities, but the identities of 22 up-regulated (Table 5) and 63 down-regulated genes (Table 6) were identified from the microarray results of day 30 post-challenge. These genes were divided into general functional categories based on GO analysis, and include those involved in binding, transport, anti-oxidant and apoptotic responses, and general immune functions (discussed below).

In addition to the commonly regulated 319 genes, one interesting finding was that unique sets of genes were found to be differentially expressed in both the heavily infected and light infected oyster groups when compared to the controls. A total of 488 genes (807 regulated genes minus 319 commonly regulated genes) were only regulated in the light infected group including 223 up-regulated and 265 down-regulated genes (Supplementary file 2); and 226 genes were only regulated in the heavily infected group including 109 up-regulated and 117 down-regulated genes (Supplementary file 3). The unique sets of genes regulated in oysters with different levels of infection are likely reflections of the dynamic expression of the host genome under various infection conditions.

4. Discussion

Oysters, while possessing a robust innate immune system, are acutely susceptible to infection by *P. marinus* trophozoites. Large gaps remain in our understanding of *P. marinus* infection routes and host immunity. Strategies aimed at producing effective treatments for dermo disease must be informed by an understanding of broader host responses and host–parasite interactions points. Towards this end, we have created and utilized a 12K oyster microarray to study regulation of gene expression following *P. marinus* infection. Micro-array-based studies have the potential to complement and extend single gene studies by capturing pathway-level gene profiles and identifying novel gene candidates for additional research. Indeed, our results, while capturing previously noted anti-oxidant/apoptosis driven responses, identified a number of additional putative immune gene processes (discussed below) that deserve further attention.

Gene ontology analysis revealed that several of the up-regulated known genes were involved in the regulation of oxidative stress and apoptosis. Three metallothioneins (ivc, iie and iif) were strongly up-regulated at 30-days post-challenge in the oysters. Metallothioneins (MT) have been identified as important regulators of both oxidative stress and apoptosis. MT gene activation induced by oxygen radicals has been observed [52,53], which may explain the possible involvement of MT in the cellular anti-oxidant defense system. First, MT shows a remarkable in vitro scavenging activity against different free-radical species [54,55]; second, the cellular level of MT can be elevated by different oxidants [20,56]; third, over expression of MT can help the cultured cells resist oxidative stress [57], whereas MT-deficient cells are more sensitive to oxidizing

Table 4
Validation of representative genes that are differentially expressed between control and treatment groups after *P. marinus* infection by quantitative PCR.

Probe	Description	Microarray fold change		qRT-PCR fold change (*P*-value)	
		Light	Heavy	Light	Heavy
Shared between light infection/heavy infection					
CV133159	Metallothionein iie	+3.43	+3.39	+6.2 (0.033)	+3.9 (0.032)
CV132138	Conserved hypothetical protein	+4.23	+3.46	+4.8 (0.047)	+4.1 (0.032)
CV087794	Metallothionein ivc	+5.09	+6.36	+7.3 (0.033)	+10.7 (0.016)
EH647818	Dynein intermediate chain 1	−3.87	−3.24	−3.1 (0.001)	−2.6 (0.016)
CD649717	TNF receptor member 19	−4.41	−5.36	−4.6 (0.016)	−3.4 (0.016)
CV088129	Ubiquitin-conjugating enzyme	−9.99	−6.13	−12.5 (0.001)	−4.7 (0.016)
Light infection only					
CV088356	C-type lectin-1	+6.09	–	+12.1 (0.032)	–
CV088207	C-type lectin 10	+4.48	–	+4.5 (0.032)	–
CD646472	Cyclin B3	−2.93	–	−4.2 (0.015)	–
Heavy infection only					
EH648615	Unknown	–	+4.70	–	+4.2 (0.033)
EH644464	Peroxiredoxin 6	–	+2.23	–	+3.6 (0.048)
CD649975	Carbonic anhydrase precursor	–	−5.05	–	−7.4 (0.001)

agents and oxidative stress [58]; and fourth, MTs were also able to prevent free-radical injury to biological structures both in vitro [59] and in vivo [60].

Oxidative stress is known to induce apoptosis in many cellular systems [16]. In vitro studies with human peripheral blood mononuclear cells from patients with acute *Plasmodium falciparum* malaria infection have also demonstrated apoptosis [61]. The role of MT in apoptosis has been investigated and the vast majority of studies show that MT plays a protective role with respect to apoptosis [62]. During in vitro studies, MT expression regulation has been correlated with resistance to apoptosis induced by doxorubicin in cardiomyocytes [63], and with metal and oxidative stress in retinal pigment cells [64]. The mechanism of apoptosis regulation of MT is likely due to direct interactions of the apoptosis-inducing agents with the MT protein leading to lower concentration of the apoptotic agent.

Several defense, immune, and apoptosis response-related genes were identified in down-regulated genes. Cathepsin S precursor [65] and Interferon-induced protein 44 were related to the immune response. A similar gene Cathepsin L1 has been characterized and found to be related in the immune response in pearl oyster [66]. Fas apoptotic inhibitory molecule [67], Rhotekin [68], Baculoviral iap repeat-containing 3 [69], Tumor necrosis factor receptor member 19 [70] and Transmembrane protein 77 [71] were differentially expressed according to the microarray results and have described functions related to the regulation of apoptosis. Moreover, within the down-regulated ion binding genes, several calcium binding genes were identified including BTD domain containing 19, EF-hand domain containing 2, EF-hand domain member b, Cox15 protein, Troponin c and Calmodulin binding protein. These calcium binding genes are involved in the maintenance of calcium homeostasis [72]. EF-hand domain containing 2 has been characterized in disc

Table 5
Up-regulated known genes from both light and heavy infection group at the 30 days after *P. marinus* challenge, as determined by microarray analysis.

GO annotation Function	Gene accession	Description	Fold change	
			Light vs Ctl	Heavy vs Ctl
Binding				
actin binding	CV088634	Profilin	4.08	5.39
metal ion binding	CV087794	Metallothionein IVC	5.09	6.36
metal ion binding	CV088743	Metallothionein IIF	3.59	3.23
metal ion binding	CV133159	Metallothionein IIe	3.43	3.39
nucleotide binding	BG624455	Histone H3.3	11.54	6.48
nucleotide binding	EH643877	ATP-binding cassette transporter sub-family a member 3	4.32	3.05
nucleotide binding	BG624428	Histone H2b	2.92	2.77
protein binding	EH648693	Rho GTPase-activating protein	3.24	3.87
protein binding	EH644443	Prolactin regulatory element-binding protein	2.63	2.70
receptor binding	EH648988	Fibrinogen c domain containing 1	3.90	4.74
Other				
	CK240404	Translocator protein	2.44	3.06
arginine kinase activity	CD646729	Arginine kinase	4.99	4.21
electron transport	CV088395	NADH dehydrogenase flavoprotein 3	2.54	3.49
regulation of transcription	EH644704	Zinc finger homeodomain 2	3.38	3.98
transmembrane transport	EH649185	Sodium-dependent glucose transporter 1	2.67	2.90
Unknown				
	CD526814	Senescence-associated protein	3.63	3.39
	CD647683	AC1147-like protein	2.46	2.47
	CD648229	NDT80-like protein	2.79	2.46
	EH647134	Merozoite-related surface protein 5	3.13	3.07
	CV132138	Conserved hypothetical protein	4.23	3.46
	EH647151	Mosaic protein LR11	4.24	3.81
	EH648184	Viral a-type inclusion protein repeat-containing protein	2.57	3.01

Table 6
Down-regulated known genes from both light and heavy infection groups after 30 days *P. marinus* infection, as determined by microarray analysis.

GO annotation Function	Gene accession	Description	Fold change	
			Light vs Ctl	Heavy vs Ctl
Ion, nucleotide, and protein binding				
calcium ion binding	EH645251	BTD domain containing 19	−4.06	−2.40
calcium ion binding	CD647305	EF-hand domain containing 2	−3.41	−2.59
calcium ion binding	EH649119	EF-hand domain member b	−3.22	−2.80
calcium ion binding	EH643958	Cox15 protein	−2.72	−2.48
calcium ion binding	EH646757	Troponin c	−2.46	−2.46
calmodulin binding	CD650655	Calmodulin binding protein, CRA_a	−3.28	−3.66
nucleotide binding	CD647385	Yeast ino80-like protein	−6.00	−4.57
nucleotide binding	EH648012	Ras-related protein Rab-35	−3.43	−2.36
nucleotide binding	EH648161	Rho family GTPase 3	−2.90	−2.36
nucleotide binding;	CD647013	Metastasis associated 1 family, member 3	−2.60	−2.99
protein binding	EH647696	Rab acceptor 1	−3.67	−2.90
protein binding	EH645734	Integrin alpha 5	−2.60	−6.04
protein complex	EH646936	Rab alpha subunit	−3.76	−2.89
Immune, defense, stress, and apoptosis				
Pathogen recognition	EH645010	*C. virginica* galectin (CvGal)	−2.78	−2.97
negative regulation of macroautophagy	EH645600	FK506-binding protein	−6.19	−2.81
immune response	EH648252	Cathepsin S precursor	−2.63	−3.45
immune response	CD648347	Interferon-induced protein 44	−2.52	−3.06
regulation of anti-apoptosis	EH644652	Fas apoptotic inhibitory molecule	−3.67	−2.62
regulation of anti-apoptosis	EH645903	Rhotekin	−2.64	−3.08
regulation of apoptosis	EH644983	Baculoviral iap repeat-containing 3	−5.45	−8.35
regulation of apoptosis	CD649717	Tumor necrosis factor receptor member 19	−4.41	−5.36
regulation of apoptosis	EH647902	Transmembrane protein 77	−3.04	−2.73
Transcription and translation				
regulation of transcription	EH645116	ATP-binding cassette transporter sub-family a member 2	−4.88	−4.04
regulation of transcription	EH646453	Trithorax protein ASH2	−2.77	−3.73
regulation of transcription	EH647103	Euchromatic histone-lysine N-methyltransferase 2	−2.77	−3.46
regulation of transcription	EH644916	SRY-box containing gene 4a	−2.58	−3.50
transcription activator activity	EH644984	Glutamate AMPA 4b	−2.63	−2.64
transcription factor activity;	BG624447	Transcription factor iib	−5.03	−3.27
translation initiation factor activity	BG624116	Eukaryotic translation initiation factor 6	−4.97	−3.04
Transport				
transmembrane transport	EH647141	Sugar transporter	−2.69	−3.24
transmembrane transport	EH647831	Solute carrier family 44 member 1	−2.47	−2.57
transmembrane transport	EH648237	Solute carrier family 37 member 2	−3.20	−3.29
transmembrane transport	EH644863	Cationic amino acid transporter	−2.89	−2.86
transmembrane transport	CD649250	Solute carrier family 6 member 5	−3.01	−5.42
transmembrane transport	EH646093	Solute carrier family 30 member 6	−4.33	−3.56
transporter activity	EH648330	Spinster homolog 1	−4.12	−3.64
transporter activity	CD648418	SEC14-like 1 (S cerevisiae)	−2.58	−2.90
Other				
amino acid activation	CD648728	Asparaginyl-tRNA synthetase	−3.33	−3.95
chaperone binding	CK240431	Activator of heat shock 90 kDa protein ATPase homolog 1	−7.56	−3.08
hemopoiesis	CV132655	Placenta growth factor	−2.56	−2.92
hormone activity	EH647044	Luteinizing hormone isoform CRA_A	−3.24	−3.93
hydrolase activity	CV087718	Endo-1,3-beta-D-glucanase	−3.98	−3.39
hydrolase activity;	EH647492	Ectonucleoside triphosphate diphosphohydrolase 1	−2.50	−2.70
ligase activity	CV088129	Ubiquitin-conjugating enzyme	−9.99	−6.13
motor activity	EH647818	Dynein intermediate chain 1	−3.87	−3.24
oligosaccharide transferase activity	EH649338	Integral membrane protein 1	−3.35	−3.05
oxidoreductase activity	CD649253	Ribonucleotide reductase m1	−5.27	−3.92
oxidoreductase activity	CD646623	Gamma hydroxybutyrate dehydrogenase	−2.72	−2.73
peptidase activity	EH645736	Similar to thymus-specific serine protease	−3.32	−2.92
ubiquitin-protein ligase activity	CD648487	Beta-transducin repeat-containing	−2.72	−3.11
ubiquitin-protein ligase activity	EH646812	Ubiquitin-conjugating enzyme E2W	−2.72	−2.37
transketolase activity	EH648695	Transketolase	−3.90	−3.38
cell differentiation	CD648429	Spermatogenesis associated 6	−4.82	−2.68
cell differentiation	EH646343	Scalloped-like transcription factor	−3.00	−2.64
regulation of cell cycle	EH649368	Cyclin g1	−3.48	−3.66
regulation of signal transduction ARF protein	EH646447	F-box protein 8	−3.01	−2.39
Unknown				
	EH646686	SFT2 domain containing 2	−8.43	−4.95
	EH647995	Kelch-like 15	−4.39	−4.19
	CD649726	UNC93a protein	−3.82	−2.87
	EH648187	Chromosome 2 open reading frame 24	−3.59	−2.92
	CD648657	Outer membrane adhesion like protein	−2.93	−2.53
	CD647938	Coiled-coil domain containing 108	−3.34	−3.18
	EH646278	Membrane palmitoylated 5	−3.03	−3.13

abalone, and the expression analysis indicated it is related to the calcium homeostasis and immune response [73].

Several lectin genes were differentially expressed in some or all groups tested in this study. C-type lectin 1, previously characterized in *C. gigas* [21], was up-regulated greater than 6-fold in light infection samples. Similarly, another lectin, not previously described in oyster, with closest similarity to yellow perch C-type lectin 10, was also strongly up-regulated in light infection samples. Further research is needed on the classes of molecules recognized by these lectins and their tissue/cell expression specificities. More is known about *C. virginica* galectin, CvGal. CvGal has been previously identified as a hemocyte receptor that preferentially recognizes *P. marinus* and that promotes phagocytosis of the infectious trophozoites. The phagocytosed trophozoites survive intrahemocytic killing, are carried into tissues and the circulatory system, and further proliferate [22]. Interestingly, in both light and heavy infection samples, we found that CvGal was down-regulated close to 3-fold compared to control samples (Table 6). This differs from the upregulation in expression reported by Tasumi and Vasta [22] following initial infection. We speculate that the down-regulation of expression of CvGal may represent a late-stage immune response to restrict further parasite uptake and intracellular proliferation. Alternatively, as CvGal also participates in homeostatic uptake of phytoplankton, the down-regulation of CvGal gene expression may represent alterations in the feeding behaviors of the infected oysters. Further work is needed to examine CvGal expression at regular intervals during the course of the *P. marinus* infection and recovery.

Among the relatively few genes with known identity that were up-regulated in both light and heavy infection samples were two histone components (Table 5). Histone H3.3 and histone H2B were up-regulated 11.54 and 2.92 fold, respectively, in light infection samples and 6.48 and 2.77 fold, respectively, in heavy infection samples. Recent research has revealed the potent antimicrobial activity of core histone proteins against fish pathogens including parasites [74]. Research using shrimp hemocytes reported antimicrobial activity for both H2B and H3 [75], indicating that these immune functions also are present in invertebrate species. In *C. virginica*, recent research [76] indicated that H2B has potent antimicrobial activity against *Vibrio* species. Our results show that H2B may also be produced in oyster gill cells in response to parasite infections, including *P. marinus*. Further follow-up studies are clearly needed to better characterize potential H2B and H3.3 antimicrobial activity against dermo and to compare antimicrobial activity between resistant and susceptible oyster strains.

Most of the differentially expressed genes identified in oysters are regulated in response to *P. marinus* infection but are probably not responsible for the active inhibition or killing of the parasite. This is supported by the observation that oysters with light and heavy infections had similar responses with little difference between them. As discussed above, the response in host is apparently part of a mechanism that, through anti-oxidation and apoptosis regulation, reduces damage to the host from ROS.

Although a large number of genes with differential expression were shared by both light and heavy infection groups, some genes with differential expression were observed in heavy infection groups but not light infection groups, and vice versa (Supplementary Tables 2 and 3). Manual inspection of these genes expression values from both groups, however, revealed that differentially expressed genes between light infection and heavy infection group had similar trends of either upregulation or down-regulation (data not shown). In the majority of cases, significant differential expression was not captured in both groups due to high gene expression variation within either light infection or heavy infection samples. Sufficient variability appeared to exist within the gene expression responses of lightly and heavily infected oysters as to make quantitative differentiation of their responses to infection very difficult.

While a large number of genes were discovered to be up-regulated or down-regulated, the majority of these genes could not be identified by BLASTX search. Further, the limited understanding of the functions of some genes identified from BLASTX searches makes it difficult to interpret comprehensive pathway regulation from the microarray results. Recently, Tanguy et al. (2008) sequenced over 10,000 ESTs in *C. gigas* and found that no more than 27% could be assigned to a functional category [77]. The whole genome sequence for *C. gigas* may be soon available, along with the identification of novel genes. This will aid in the understanding of their functions, not only in oysters, but also other mollusks. We expect that this dataset will continue to yield insights into the pathways that can unravel the host parasite interaction and provide some strategies to minimize the effects of disease.

In conclusion, a 12K oligo microarray was developed that allowed analysis of differentially expressed genes in eastern oyster after parasite infection. Microarray analysis revealed that a large number of genes were differentially expressed after the parasite infection regardless of light infection or heavy infection, and many of the known differentially expressed genes were involved in pathogen recognition, antimicrobial defenses, oxidative stress and apoptosis regulation. Significant differential expression was not observed between light infection and heavy infection groups. Further studies are required to understand the difference in gene expression responses between resistant and susceptible oysters and to compare these results to those obtained from naturally occurring parasitic infections observed in wild oysters.

Acknowledgements

This project was supported by a grant from NOAA Sea Grant Gulf Oyster Industry Program awarded to ZL, RW, and XG. I Burt, E Scarpa and others at HSRL provided assistance with challenge experiments.

Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fsi.2010.07.035.

References

[1] Carriker MR, Gaffney PM. A catalogue of selected species of living oysters (Ostreacea) of the world. In: Kennedyk VS, Newell RIE, Eble AF, editors. The Eastern oyster *Crassostrea virginica*. USA: Maryland Sea Grant College; 1996. p. 1–18.
[2] Newell RIE, Cornwell JC, Owens MS. Influence of simulated bivalve biodeposition and microphytobenthos on sediment nitrogen dynamics: a laboratory study. Waco, TX, ETATS-UNIS: American Society of Limnology and Oceanography; 2002.
[3] Lannig G, Flores JF, Sokolova IM. Temperature-dependent stress response in oysters, *Crassostrea virginica*: pollution reduces temperature tolerance in oysters. Aquat Toxicol 2006;79:278–87.
[4] Fisher WS, Oliver LM, Winstead JT, Long ER. A survey of oysters *Crassostrea virginica* from Tampa Bay, Florida: associations of internal defense measurements with contaminant burdens. Aquat Toxicol 2000;51:115–38.
[5] Oliver LM, Fisher WS, Winstead JT, Hemmer BL, Long ER. Relationships between tissue contaminants and defense-related characteristics of oysters (*Crassostrea virginica*) from five Florida bays. Aquat Toxicol 2001;55:203–22.
[6] Lenihan HS, Peterson CH. How habitat degradation through fishery disturbance enhances impacts of hypoxia on oyster reefs. Ecol Appl 1998;8:128–40.
[7] Jackson JB, Kirby MX, Berger WH, Bjorndal KA, Botsford LW, Bourque BJ, et al. Historical overfishing and the recent collapse of coastal ecosystems. Science 2001;293:629–37.
[8] Barber BJ, Mann R. Growth and mortality of eastern oysters, *Crassostrea virginica* (Gmelin, 1791), and Pacific oysters, *Crassostrea gigas* (Thunberg, 1793) under challenge from the parasite, *Perkinsus marinus*. J Shellfish Res 1994;13:109–14.
[9] Perkins FO. *Dermocystidium marinum* infection in oysters. Mar Fish Rev NMFS 1976;38:19–21.
[10] Levine ND. *Perkinsus gen.* n. and other new taxa in the protozoan phylum Apicomplexa. J Parasitol 1978;64:549.

[11] Ford SE, Tripp MR. Diseases and defense mechanisms in the Eastern Oyster *Crassostrea virginica*. MD, USA: Maryland Sea Grant, College Park; 1996.
[12] Burreson EM, Ragone Calvo LM. Epizootiology of *Perkinsus marinus* disease of oysters in Chesapeake Bay, with emphasis on data since 1985. J Shellfish Res 1996;15:17–34.
[13] Anderson RS. Interactions of *Perkinsus marinus* with humoral factors and hemocytes of *Crassostrea virginica*. J Shellfish Res 1996;15:127–34.
[14] Adema CM, Vanderknapp WP, Sminia T. Molluscan hemocyte-mediated cytotoxicity: the role of reactive oxygen intermediates. Rev Aquat Sci 1991;4:201–23.
[15] Anderson RS, Oliver LM, Brubacher LL. Superoxide anion generation by *Crassostrea virginica* hemocytes as measured by nitrotetrazolium reduction. J Invertebr Pathol 1992;59:303–7.
[16] Guha M, Kumar S, Choubey V, Maity P, Bandyopadhyay U. Apoptosis in liver during malaria: role of oxidative stress and implication of mitochondrial pathway. FASEB J 2006;20:1224–6.
[17] Goedken M, Morsey B, Sunila I, De Guise S. Immunomodulation of *Crassostrea gigas* and *Crassostrea virginica* cellular defense mechanisms by *Perkinsus marinus*. J Shellfish Res 2005;24:487–96.
[18] Hughes FM, Foster B, Grewal S, Sokolova IM. Apoptosis as a host defense mechanism in *Crassostrea virginica* and its modulation by *Perkinsus marinus*. Fish Shellfish Immunol 2010;29:247–57.
[19] Viarengo A, Burlando B, Ceratto N, Panfoli I. Antioxidant role of metallothioneins: a comparative overview. Cell Mol Biol (Noisy-le-grand) 2000;46:407–17.
[20] Anderson RS, Patel KM, Roesijadi G. Oyster metallothionein as an oxyradical scavenger: implications for hemocyte defense responses. Dev Comp Immunol 1999;23:443–9.
[21] Yamaura K, Takahashi KG, Suzuki T. Identification and tissue expression analysis of C-type lectin and galectin in the Pacific oyster, *Crassostrea gigas*. Comp Biochem Physiol B Biochem Mol Biol 2008;149:168–75.
[22] Tasumi S, Vasta GR. A galectin of unique domain organization from hemocytes of the Eastern oyster (*Crassostrea virginica*) is a receptor for the protistan parasite *Perkinsus marinus*. J Immunol 2007;179:3086–98.
[23] Jenny MJ, Chapman RW, Mancia A, Chen YA, McKillen DJ, Trent H, et al. A cDNA microarray for *Crassostrea virginica* and *C. gigas*. Mar Biotechnol (NY) 2007;9:577–91.
[24] Peatman E, Baoprasertkul P, Terhune J, Xu P, Nandi S, Kucuktas H, et al. Expression analysis of the acute phase response in channel catfish (*Ictalurus punctatus*) after infection with a Gram-negative bacterium. Dev Comp Immunol 2007;31:1183–96.
[25] Peatman E, Terhune J, Baoprasertkul P, Xu P, Nandi S, Wang S, et al. Microarray analysis of gene expression in the blue catfish liver reveals early activation of the MHC class I pathway after infection with *Edwardsiella ictaluri*. Mol Immunol 2008;45:553–66.
[26] Wang Y, Guo X. Development and characterization of EST-SSR markers in the eastern oyster *Crassostrea virginica*. Mar Biotechnol 2007;9:500–11.
[27] Yu H, Li Q. Genetic variation of wild and hatchery populations of the pacific oyster *Crassostrea gigas* assessed by microsatellite markers. J Genet Genomics 2007;34:1114–22.
[28] Wang Y, Ren R, Yu Z. Bioinformatic mining of EST-SSR loci in the Pacific oyster, *Crassostrea gigas*. Anim Genet 2008;39:287–9.
[29] Yu H, Li Q. Exploiting EST databases for the development and characterization of EST-SSRs in the Pacific oyster (*Crassostrea gigas*). J Hered 2008; 99:208–14.
[30] Yu Z, Guo X. Genetic linkage map of the eastern oyster *Crassostrea virginica* Gmelin. Biol Bull 2003;204:327–38.
[31] Cunningham C, Hikima J, Jenny MJ, Chapman RW, Fang GC, Saski C, et al. New resources for marine genomics: bacterial artificial chromosome libraries for the Eastern and Pacific oysters (*Crassostrea virginica* and *C. gigas*. Mar Biotechnol (NY) 2006;8:521–33.
[32] Jenny MJ, Ringwood AH, Lacy ER, Lewitus AJ, Kempton JW, Gross PS, et al. Potential indicators of stress response identified by expressed sequence tag analysis of hemocytes and embryos from the American oyster, *Crassostrea virginica*. Mar Biotechnol (NY) 2002;4:81–93.
[33] Peatman EJ, Wei X, Feng J, Liu L, Kukuktas H, Li P, et al. Development of expressed sequence tags from eastern oyster (*Crassostrea virginica*): lessons learned from previous efforts. Mar Biotechnol 2004;6:S491–6.
[34] Tanguy A, Guo X, Ford SE. Discovery of genes expressed in response to *Perkinsus marinus* challenge in Eastern (*Crassostrea virginica*) and Pacific (*C. gigas*) oysters. Gene 2004;338:121–31.
[35] Quilang J, Wang S, Li P, Abernathy J, Peatman E, Wang Y, et al. Generation and analysis of ESTs from the eastern oyster, *Crassostrea virginica* Gmelin and identification of microsatellite and SNP markers. BMC Genomics 2007; 8:157.
[36] Sauvage C, Bierne N, Lapègue S, Boudry P. Single Nucleotide polymorphisms and their relationship to codon usage bias in the Pacific oyster *Crassostrea gigas*. Gene 2007;406:13–22.
[37] Roberts S, Goetz G, White S, Goetz F. Analysis of genes isolated from plated hemocytes of the Pacific oyster, *Crassostreas gigas*. Mar Biotechnol (NY) 2009;11:24–44.
[38] Lang RP, Bayne CJ, Camara MD, Cunningham C, Jenny MJ, Langdon CJ. Transcriptome profiling of selectively bred Pacific oyster *Crassostrea gigas* families that differ in tolerance of heat shock. Mar Biotechnol (NY) 2009;11:650–68.
[39] Ford SE, Chintala MM, Bushek D. Comparison of in vitro-cultured and wild type *Perkinsus marinus*. I. Pathogen virulence. Dis Aquat Org 2002;51:187–201.
[40] Bushek D, Ford SE, Allen Jr SK. Evaluation of methods using ray's fluid thioglycollate medium for diagnosis of *Perkinsus marinus* infection in the eastern oyster, *Crassostrea virginica*. Annu Rev Fish Dis 1994;4:201–17.
[41] Choi KS, Wilson EA, Lewes DH, Powell EN, Ray SM. The energetic cost of *Perkinsus marinus* parasitism in oysters: quantification of the thioglycollate methods. J Shellfish Res 1989;8:125–31.
[42] Huang X, Madan A. CAP3: a DNA sequence assembly program. Genome Res 1999;9:868–77.
[43] Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 2003;19:185–93.
[44] Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 2003;4:249–64.
[45] Li C. Automating dChip: toward reproducible sharing of microarray data analysis. BMC Bioinform 2008;9:231.
[46] Kalendar R. FastPCR: a PCR primer and probe design and repeat sequence searching software with additional tools for the manipulation and analysis of DNA and protein. 2008.
[47] Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:e45.
[48] Jorgensen SM, Kleveland EJ, Grimholt U, Gjoen T. Validation of reference genes for real-time polymerase chain reaction studies in Atlantic salmon. Mar Biotechnol (NY) 2006;8:398–408.
[49] Zhong Q, Zhang Q, Wang Z, Qi J, Chen Y, Li S, et al. Expression profiling and validation of potential reference genes during *Paralichthys olivaceus* embryogenesis. Mar Biotechnol (NY) 2008;10:310–8.
[50] Fleury E, Moal J, Boulo V, Daniel JY, Mazurais D, Henaut A, et al. Microarray-based identification of gonad transcripts differentially expressed between lines of pacific oyster selected to be resistant or susceptible to summer mortality. Mar Biotechnol (NY) 2009;12:326–39.
[51] Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 2000;25:25–9.
[52] Sato M, Bremner I. Oxygen free radicals and metallothionein. Free Radic Biol Med 1993;14:325–37.
[53] Kelley K, Edward K. Antioxidant function of metallothioneins. J Trace Elem Exp Med 1998;11:219–26.
[54] Thornalley PJ, Vasak M. Possible role for metallothionein in protection against radiation-induced oxidative stress. Kinetics and mechanism of its reaction with superoxide and hydroxyl radicals. Biochim Biophys Acta 1985;827:36–44.
[55] Kumari MV, Hiramatsu M, Ebadi M. Free radical scavenging actions of metallothionein isoforms I and II. Free Radic Res 1998;29:93–101.
[56] Bauman JW, Liu J, Liu YP, Klaassen CD. Increase in metallothionein produced by chemicals that induce oxidative stress. Toxicol Appl Pharmacol 1991;110: 347–54.
[57] Schwarz MA, Lazo JS, Yalowich JC, Reynolds I, Kagan VE, Tyurin V, et al. Cytoplasmic metallothionein overexpression protects NIH 3T3 cells from tert-butyl hydroperoxide toxicity. J Biol Chem 1994;269:15238–43.
[58] Lazo JS, Kondo Y, Dellapiazza D, Michalska AE, Choo KH, Pitt BR. Enhanced sensitivity to oxidative stress in cultured embryonic cells from transgenic mice deficient in metallothionein I and II genes. J Biol Chem 1995;270:5506–10.
[59] Abel J, de Ruiter N. Inhibition of hydroxyl-radical-generated DNA degradation by metallothionein. Toxicol Lett 1989;47:191–6.
[60] Mimura T, Tsujikawa K, Yasuda N, Nakajima H, Haruyama M, Ohmura T, et al. Suppression of gastric ulcer induced by stress and HCL-ethanol by intravenously administered metallothionein-II. Biochem Biophys Res Commun 1988;151:725–9.
[61] Toure-Balde A, Sarthou JL, Aribot G, Michel P, Trape JF, Rogier C, et al. *Plasmodium falciparum* induces apoptosis in human mononuclear cells. Infect Immun 1996;64:744–50.
[62] Shimoda R, Achanzar WE, Qu W, Nagamine T, Takagi H, Mori M, et al. Metallothionein is a potential negative regulator of apoptosis. Toxicol Sci 2003;73:294–300.
[63] Wang GW, Zhou Z, Klein JB, Kang YJ. Inhibition of hypoxia/reoxygenation-induced apoptosis in metallothionein-overexpressing cardiomyocytes. Am J Physiol Heart Circ Physiol 2001;280:2292–9.
[64] Lu H, Hunt DM, Ganti R, Davis A, Dutt K, Alam J, et al. Metallothionein protects retinal pigment epithelial cells against apoptosis and oxidative stress. Exp Eye Res 2002;74:83–92.
[65] Shi GP, Webb AC, Foster KE, Knoll JH, Lemere CA, Munger JS, et al. Human cathepsin S: chromosomal localization, gene structure, and tissue distribution. J Biol Chem 1994;269:11530–6.
[66] Ma J, Zhang D, Jiang J, Cui S, Pu H, Jiang S. Molecular characterization and expression analysis of cathepsin L1 cysteine protease from pearl oyster Pinctada fucata. Fish Shellfish Immunol 2010;29:501–7.
[67] Segura MF, Sole C, Pascual M, Moubarak RS, Perez-Garcia MJ, Gozzelino R, et al. The long form of Fas apoptotic inhibitory molecule is expressed specifically in neurons and protects them against death receptor-triggered apoptosis. J Neurosci 2007;27:11228–41.
[68] Liu CA, Wang MJ, Chi CW, Wu CW, Chen JY. Rho/Rhotekin-mediated NF-kappaB activation confers resistance to apoptosis. Oncogene 2004;23:8731–42.

[69] Liston P, Roy N, Tamai K, Lefebvre C, Baird S, Cherton-Horvat G, et al. Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. Nature 1996;379:349–53.
[70] Eby MT, Jasmin A, Kumar A, Sharma K, Chaudhary PM. TAJ, a novel member of the tumor necrosis factor receptor family, activates the c-Jun N-terminal kinase pathway and mediates caspase-independent cell death. J Biol Chem 2000;275:15336–42.
[71] Park SM, Kim K, Lee EJ, Kim BK, Lee TJ, Seo T, et al. Reduced expression of DRAM2/TMEM77 in tumor cells interferes with cell death. Biochem Biophys Res Commun 2009;390:1340–4.
[72] Ikura M. Calcium binding and conformational response in EF-hand proteins. Trends Biochem Sci 1996;21:14–7.
[73] Nikapitiya C, De Zoysa M, Whang I, Kim SJ, Choi CY, Lee JS, et al. Characterization and expression analysis of EF hand domain-containing calcium-regulatory gene from disk abalone: Calcium homeostasis and its role in immunity. Fish Shellfish Immunol 2010;29:334–42.
[74] Noga EJ, Fan Z, Silphaduang U. Histone-like proteins from fish are lethal to the parasitic dinoflagellate *Amyloodinium ocellatum*. Parasitology 2001;123:57–65.
[75] Patat SA, Carnegie RB, Kingsbury C, Gross PS, Chapman R, Schey KL. Antimicrobial activity of histones from hemocytes of the Pacific white shrimp. Eur J Biochem 2004;271:4825–33.
[76] Seo JK, Stephenson J, Crawford JM, Stone KL, Noga EJ. American oyster, Crassostrea virginica, expresses a potent antibacterial histone H2B protein. Mar Biotechnol, doi:10.1007/s10126-009-9240-z.
[77] Tanguy A, Bierne N, Saavedra C, Pina B, Bachere E, Kube M, et al. Increasing genomic information in bivalves through new EST collections in four species: development of new genetic markers for environmental studies and genome evolution. Gene 2008;408:27–36.